Physiological concentrations of inorganic phosphate affect MgATP-dependent Ca^{2+} storage and inositol trisphosphate-induced Ca^{2+} efflux in microsomal vesicles from non-hepatic cells

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1. MgATP-dependent $^{45}\text{Ca}^{2+}$ uptake by microsomes obtained from various non-hepatic tissues, namely rat brain, rat solid Morris hepatoma 3924A and human platelets, was measured in the presence of P_i at low, cytosol-like, concentrations. 2. Increasing P_i concentrations (0.5–3 mM) caused a progressive enlargement of the $^{45}\text{Ca}^{2+}$ -storage capacity of all the microsomal fractions. 3. As a result of P_i stimulation of Ca^{2+} uptake, $^{45}\text{Ca}^{2+}$ and $[^{32}\text{P}]\text{P}_i$ were co-accumulated by the three microsomal fractions. 4. The time course for $^{45}\text{Ca}^{2+}$ and $[^{32}\text{P}]\text{P}_i$ accumulation in brain microsomes revealed a biphasic $^{45}\text{Ca}^{2+}$ uptake: a rapid phase was followed by a second, slower, phase, which depended on the presence of P_i . During the P_i -dependent phase, the uptake of $^{45}\text{Ca}^{2+}$ was paralleled by the uptake of $[^{32}\text{P}]\text{P}_i$. 5. The passive efflux of Ca^{2+} was paralleled by the efflux

of P_i and vice versa. In fact, the inhibition of active Ca^{2+} uptake by excess EGTA, or lowering the P_i concentration of the incubation system by dilution, caused the release of $^{45}\text{Ca}^{2+}$ and $[^{32}\text{P}]\text{P}_i$ from $^{45}\text{Ca}^{2+}$ or $[^{32}\text{P}]\text{P}_i$ pre-loaded brain microsomes. The Ca^{2+} ionophore A23187 also released $^{45}\text{Ca}^{2+}$ and $[^{32}\text{P}]\text{P}_i$. 6. Ca^{2+} efflux by A23187 was rapid ($t_{1/2}$ approx. 2 s) and independent of the extent of intravesicular Ca^{2+} loading, which indicates that Ca^{2+} and P_i do not form intravesicular insoluble complexes. 7. The progressive increase in Ca^{2+} accumulation, depending on P_i stimulation, resulted in a proportional increase in the amount of Ca^{2+} releasable by InsP_3 in the three non-hepatic microsomal fractions and in digitonin-permeabilized platelets. 8. Concomitantly to Ca^{2+} , microsomal P_i was also released by InsP_3 .

INTRODUCTION

It is well established that the endoplasmic reticulum (ER) of non-muscle cells, like the analogous sarcoplasmic reticulum in muscle, possesses a MgATP-dependent Ca^{2+} pump(s), and thus the ER plays an important role in the regulation of intracellular levels of Ca^{2+} ions. Indeed, this organelle appears to comprise the intracellular Ca^{2+} pool responsive to the InsP_3 -mediated agonists [1–6] and to regulate its own degree of Ca^{2+} filling, as this modulates the entry of extracellular Ca^{2+} via a putative unidentified mediator(s) [7].

Although the ER is believed to be a major Ca^{2+} -storage site *in vivo* [8–10], microsomal fractions (i.e. vesicles derived principally from ER) obtained from various non-muscle cells exhibit a relatively low Ca^{2+} accumulating capacity (along with a high Ca^{2+} affinity) [3,4]. This low Ca^{2+} -sequestering capacity has been often enlarged by using intravesicular Ca^{2+} -trapping agents, such as oxalate (see [11,12] for references) or P_i (e.g. [13–15]; see also [11] for refs.), as a tool to minimize the passive Ca^{2+} back-flux and to give evidence of the MgATP-dependent Ca^{2+} inward pumping.

Searching for physiological mechanisms operative in favouring Ca^{2+} storage in the ER, we have previously shown that cytosol-like concentrations of glucose 6-phosphate [5,16] or P_i [17] are able to increase Ca^{2+} accumulation in various liver reticular preparations. In both instances, Ca^{2+} and P_i ions were co-accumulated and, in the case of glucose 6-phosphate, the hydrolysis of glucose 6-phosphate by glucose-6-phosphatase within the microsomal lumen is the source of co-accumulated P_i [16]. The latter mechanism probably operates also in non-hepatic cells which share with liver high levels of reticular glucose-6-

phosphatase, namely kidney [18] and pancreatic β -cells [19]. Indeed, reticular preparations from these tissues exhibit glucose 6-phosphate stimulation of active Ca^{2+} accumulation [18,19]. Moreover, in skeletal-muscle reticular fractions, other authors showed that low physiological concentrations of P_i cause a large Ca^{2+} loading [20,21] by forming a soluble complex with luminal Ca^{2+} [21].

Taken together, these results might suggest a major role for P_i anions in enlarging the endo(sarco)plasmic-reticulum Ca^{2+} stores. The aim of the present work was to investigate further and to clarify the role for cytosolic P_i anions in intracellular Ca^{2+} handling by the reticular network. We therefore investigated the effect of P_i on microsomal Ca^{2+} fluxes in three different non-muscle model tissues possessing no or minor glucose-6-phosphatase activity [22–24], such as rat brain, rat solid Morris hepatoma 3924A and human platelets. To this end, isolated microsomal fractions and digitonin-permeabilized platelets were challenged with P_i concentrations in the range of those reported to occur in all the tissues so far investigated [25–30].

A main result of the present study was that low physiological concentrations of P_i (0.5–3.0 mM) enlarge both reticular Ca^{2+} -loading capacity and InsP_3 -mobilizable microsomal Ca^{2+} pool(s) in the three tissues investigated.

EXPERIMENTAL

Materials

ATP, phosphocreatine, creatine kinase (Sigma type III), A23187 and digitonin were from Sigma. $^{45}\text{CaCl}_2$ (1650 Ci/mol), $\text{Na}_2\text{H}^{32}\text{PO}_4$ (1100 Ci/mol) and $[\text{G}-^3\text{H}]\text{inulin}$ (500 mCi/g) were from DuPont–New England Nuclear. $\text{Ins}(1,4,5)\text{P}_3$ was from

Boehringer. All other chemicals were of analytical grade. Ca^{2+} electrodes were purchased from Ionetics Inc., Palo Alto, CA, U.S.A. Male Sprague-Dawley rats weighing 180–220 g were purchased from Nossan, Milan, Italy. Rats bearing the solid Morris hepatoma 3924A were kindly supplied by Dr. T. Galeotti, Istituto di Patologia Generale, Università Cattolica del Sacro Cuore, Rome, Italy. Human platelet concentrates prepared for clinical transfusions were supplied by the Centro di Ematologia, Siena, Italy.

Preparation of microsomal fractions and permeabilized platelets

Platelets were isolated from platelet-rich plasma as reported in [31]. Microsomal fractions were isolated from rat brain, rat solid Morris hepatoma 3924A and human platelets by differential centrifugation as reported in refs. [32], [16] and [33] respectively. All the microsomal fractions were washed with the same medium [composition (mM): KCl, 100; NaCl, 20; MgCl_2 , 5; Mops, 20; pH 7.2] by centrifuging at 120 000 *g* for 60 min. The resulting microsomal pellets were resuspended in the above medium at a protein concentration in the range 20–100 mg/ml. The suspensions were frozen and maintained under liquid N_2 until work-up.

Human platelets were permeabilized with digitonin as reported in [17], resuspended in the KCl/Mops medium as above (approx. 150 mg of protein/ml), maintained at 0–4 °C and used within 2 h.

Morphological characteristics and intravesicular water space of the microsomal fractions

For electron-microscopy examination, the microsomal suspensions were fixed and stained as reported in [5].

Brain microsomal fraction mainly consisted of vesicles of various sizes (approx. 100–200 nm); the smooth vesicles were present in larger amounts (approx. 4-fold), smaller and more uniform in size than the rough ones. Some of the smooth vesicles included also smallest vesicles. A few particles larger than ribosomes and few membrane fragments were present. Mitochondria and mitochondrial fragments were absent.

The microsomal fraction from solid Morris hepatoma mainly comprised vesicles ranging between approx. 100 and 300 nm, which were surrounded by a large number of ribosomes. Aggregates of small vesicles as well as of ribosomes were often observed. Mitochondria and mitochondrial fragments were absent.

Platelet microsomal fraction was found to consist primarily of empty vesicles of variable size (from approx. 100 to 250 nm). Elongated profiles or tubules were also present. Rare dense bodies and granules and no mitochondria were present.

The intravesicular water space was measured by using ^3H -labelled inulin [12]. Values ($\mu\text{l}/\text{mg}$ of protein; means $\pm$ S.E.M., $n = 4$ –6) were 4.0 ± 0.6 , 4.9 ± 1.2 and 6.1 ± 0.7 in brain, hepatoma and platelet microsomes respectively.

Measurement of $^{45}\text{Ca}^{2+}$ accumulation by microsomal fractions

The microsomal fractions were incubated at 37 °C, in a medium with the following composition (mM): KCl, 100; NaCl, 20; MgCl_2 , 5; Mops, 20 (pH 7.2); ATP, 3; phosphocreatine, 10; NaN_3 (as mitochondrial inhibitor), 5. Creatine kinase (10 units/ml) was also present. CaCl_2 (20 μM final concn.) with $^{45}\text{CaCl}_2$ as a tracer (1.1–1.2 $\mu\text{Ci}/\text{ml}$) was added to the medium. Where indicated in the individual experiments, a small volume of potassium phosphate buffer, pH 7.2, was included in the in-

cubation medium to obtain the desired final concentrations of P_i . The incubation started by adding a small volume (less than 1/50 of the volume of the incubation mixture) of the microsomal suspension to the prewarmed (5 min at 37 °C) complete medium to give 0.020–0.025 mg of microsomal protein/ml. The total Ca^{2+} present in the incubation medium (Ca^{2+} added plus Ca^{2+} already present as contaminant in reagents) was measured by atomic-absorption spectroscopy in each experiment, and was in the range 30–40 nmol/ml. The amount of Ca^{2+} accumulated by microsomes was calculated on the basis of the actual total Ca^{2+} content of each incubate. Ca^{2+} accumulated by microsomes was measured by using a rapid-filtration technique [16] and corrected for the non-specifically bound Ca^{2+} , i.e. Ca^{2+} associated with microsomes in the absence of ATP.

Measurement of ^{32}P -labelled phosphates accumulated by microsomal fractions

The amount of P_i co-accumulated with Ca^{2+} by microsomal fractions was measured by adding [^{32}P] P_i to incubation systems identical with those used for measuring $^{45}\text{Ca}^{2+}$ accumulation, but in the absence of $^{45}\text{Ca}^{2+}$. [^{32}P] P_i associated with (or accumulated by) microsomes was determined by the rapid-filtration technique used to determine microsomal $^{45}\text{Ca}^{2+}$ (see above).

Measurement of Ca^{2+} fluxes with Ca^{2+} electrodes

Microsomes (0.5–2 mg of protein/ml) or permeabilized cells (3–4 mg of protein/ml) were incubated in a thermostatically controlled (37 °C) Plexiglas vessel in which a Ca^{2+} electrode and a reference electrode (Radiometer K4040) were immersed. The incubation medium (1 ml) was the following (mM): KCl, 100; NaCl, 20; MgCl_2 , 5; Mops, 20 (pH 7.2); ATP, 3; phosphocreatine, 10; NaN_3 , 5. Creatine kinase (10 units/ml) was also present. Different amounts of CaCl_2 (10–50 $\mu\text{mol}/\text{ml}$) were included in the medium in order to load microsomal vesicles with different amounts of Ca^{2+} . P_i was added to the incubation medium as potassium phosphate buffer, pH 7.2. The amount of Ca^{2+} accumulated or released by InsP_3 and by A23187 was quantified by adding several pulses of CaCl_2 (5 μM each) to parallel incubations. The Ca^{2+} electrodes were calibrated as described by others [34].

Other analytical procedures

Protein was determined by the Lowry method [35]. P_i content of incubation was measured by using a colorimetric method suitable to measure P_i in the presence of acid-labile organic phosphate (i.e. phosphocreatine) as reported by others [36].

RESULTS

Stimulation of active Ca^{2+} accumulation by phosphate in microsomal fractions from various non-hepatic tissues

The stimulatory effect of P_i on active Ca^{2+} accumulation by the various microsomal fractions is shown in Figure 1. When incubated in the presence of MgATP, microsomes rapidly (< 5 min) accumulated external $^{45}\text{Ca}^{2+}$, reaching steady-state levels which were maintained until the latest incubation time. The inclusion of P_i (0.5–3.0 mM; Figure 1, ●) in the incubation system resulted in higher values of Ca^{2+} accumulation in all the investigated fractions. In the presence of P_i , $^{45}\text{Ca}^{2+}$ uptake was biphasic: a first rapid phase was followed by a second, slower, and more prolonged, uptake phase. A biphasic pattern of P_i -stimulated $^{45}\text{Ca}^{2+}$ uptake was previously described in liver

Figure 1 Time course of MgATP-dependent Ca^{2+} uptake by brain, hepatoma and platelet microsomes in the presence or absence of P_i

The microsomal fractions (0.02–0.025 mg of protein/ml) were incubated at 37 °C, in a medium with the following composition (mM): KCl, 100; NaCl, 20; $MgCl_2$, 5; Mops, 20 (pH 7.2); ATP, 3; phosphocreatine, 10; NaN_3 (as mitochondrial inhibitor), 5. Creatine kinase (10 units/ml) was also present. $CaCl_2$ (20 μ M final concn.) with $^{45}CaCl_2$ as a tracer (1.1–1.2 μ Ci/ml) was added to the medium. Where indicated, P_i (as potassium phosphate buffer, pH 7.2) was included in the incubation medium to obtain the desired final concentrations. At the indicated time points, samples of the incubation mixture were taken to measure $^{45}Ca^{2+}$ taken up by microsomes, and Ca^{2+} uptake was calculated as reported in the Experimental section. A typical set of experiments out of 3–6 is reported.

reticular preparations [17] and in skeletal-muscle microsomes [20,21].

In these experiments (Figure 1), a low concentration of microsomal protein (about 20–25 μ g/ml) was employed to minimize the stimulatory effect of P_i derived from microsomal ATP hydrolysis and to show the effect of added P_i . Actually, in the presence of 20–25 μ g of microsomal protein the hydrolysis of ATP supplied P_i to the system at a rate of approx. 0.017, 0.014 and 0.005 mM/min in brain, hepatoma and platelets respectively. P_i production was linear over 60 min of incubation and independent of the concentration of added P_i (0–3 mM). However, such amounts of ATP-derived P_i by itself did not appear to stimulate $^{45}Ca^{2+}$ accumulation. In fact, in the presence of lower microsomal protein concentrations (20–5 μ g/ml) similar $^{45}Ca^{2+}$ -uptake curves were observed independently of the proportionally lower P_i supply (results not shown). On the other hand, with microsomal protein concentrations higher than 25–50 μ g/ml a biphasic uptake of $^{45}Ca^{2+}$ was evident and appeared to be related

to ATP-derived P_i . In fact, on increasing the protein concentration (50–200 μ g/ml) the rate of Ca^{2+} uptake of the second (P_i -dependent) phase increased proportionally, and strictly correlated with the increase in P_i supply in any microsomal fraction employed (results not shown).

The contribution of organelles other than the ER to Ca^{2+} accumulation in the different microsomal fractions appeared to be negligible. In fact, P_i -stimulated and -unstimulated Ca^{2+} accumulations were not modified in the presence of a mitochondrial inhibitor (5 mM NaN_3 , which was routinely included in the incubation system, or 2 μ M carbonyl cyanide *p*-fluoromethoxyphenylhydrazone) and in the presence of digitonin at concentrations suitable to permeabilize plasma-membrane-derived vesicles [37]. Also, the reticular Ca^{2+} -pump inhibitor thapsigargin (1 μ M; [38]) suppressed any Ca^{2+} accumulation by hepatoma and platelet microsomes, and 70 % of Ca^{2+} accumulation by brain microsomes. In brain microsomes, the incomplete inhibition of Ca^{2+} uptake by thapsigargin has been also described by others [39].

The stimulatory effect of P_i was also evident in the presence of extravesicular low free Ca^{2+} concentrations comparable with the cytosolic ones of resting and stimulated cells. In fact, in incubation systems whose free Ca^{2+} concentration was adjusted at different values (2.5–0.2 μ M, as measured by Ca^{2+} electrodes), the pattern of P_i -stimulated $^{45}Ca^{2+}$ uptake by the microsomal fractions was very similar (see Figure 2, brain microsomes). Moreover, the monitoring of MgATP-dependent Ca^{2+} uptake by Ca^{2+} electrodes revealed that after (brain) microsomes have had their external free Ca^{2+} concentration lowered to approx. 0.2 μ M, the addition of Ca^{2+} was followed by a rapid re-lowering of ambient free Ca^{2+} provided that P_i (3 mM) was included in the system (Figure 2b, continuous trace). In the absence of added P_i (Figure 2b, broken trace) re-lowering of ambient free Ca^{2+} was slower but still evident. Since in Ca^{2+} -electrode measurements we used relatively high concentrations (1 mg/ml) of microsomal protein, P_i supplied to the system by ATP hydrolysis may contribute to the Ca^{2+} -re-lowering phase.

The maximal Ca^{2+} -loading capacity of the different microsomal fractions is reported in Figure 3; it increased linearly on increasing the P_i concentration in the incubation system (0.5–3.0 mM). Values for Ca^{2+} accumulation were also expressed on the basis of the intravesicular water space of the different microsomal fractions (Figure 3b) in order to calculate the intravesicular concentrations of 'total' Ca^{2+} . Under P_i stimulation, these concentrations were between 5 and 30 mM in the three non-hepatic tissues.

Additional experiments were performed with digitonin-permeabilized platelets incubated in the presence of mitochondrial inhibitors to verify the behaviour of the unfragmented ER *in situ*. In the absence of added P_i , the maximal Ca^{2+} -loading capacity of the non-mitochondrial platelet pool(s) was 2.42 ± 0.40 nmol/60 min per mg of protein (mean $\pm$ S.E.M., $n = 3$). It increased to 23.40 ± 2.40 nmol/60 min per mg of protein in the presence of 3.0 mM P_i .

P_i -stimulated Ca^{2+} uptake was accompanied by an attendant co-accumulation of P_i in microsomal vesicles. As shown in Table 1, the addition of P_i to the incubation system enlarged the $^{45}Ca^{2+}$ -loading capacity of the microsomal fractions. Concomitantly, radiolabelled P_i were also accumulated, as revealed in parallel samples including [^{32}P] P_i (instead of $^{45}Ca^{2+}$). Net values for microsomal [^{32}P] P_i accumulation were obtained by subtracting [^{32}P] P_i non-specifically associated with microsomes, i.e. in the absence of any active Ca^{2+} accumulation. The latter was suppressed by including A23187 (2 μ M) in parallel samples. Similar values for [^{32}P] P_i non-specifically associated with

Figure 2 MgATP-dependent $^{45}\text{Ca}^{2+}$ uptake (a) and buffering capacity (b) of brain microsomes incubated in the presence or in the absence of P_i in low-free- Ca^{2+} media

(a) Incubation conditions for MgATP-dependent $^{45}\text{Ca}^{2+}$ uptake by microsomes were as reported in the legend in Figure 1, except that Mg^{2+} was lowered from 5 to 1 mM to give a higher ATP/ Mg^{2+} ratio suitable for decreasing the free Ca^{2+} concentration in the incubation systems. No CaCl_2 (●), 20 μM CaCl_2 (□) or 90 μM CaCl_2 (△) was included in the system. Total Ca^{2+} already present as contaminant in reagents, in the absence of added CaCl_2 was 14.3 μM . At the beginning of incubation pCa values of the incubation were measured by Ca^{2+} electrodes, and are indicated in panel (a). No (broken lines) or 3 mM (continuous lines) P_i was included in the incubation medium. A typical experiment out of three is reported. (b) Microsomal Ca^{2+} uptake was measured by monitoring the free Ca^{2+} concentration in the incubation mixture with Ca^{2+} electrodes. Brain microsomes (MS; 2 mg of protein/ml) were incubated in the presence of MgATP at 37 °C under continuous stirring. Total Ca^{2+} already present, as contaminant in reagents, before any CaCl_2 additions was 13.2 μM . No (broken line) or 3 mM (continuous line) P_i was included in the incubation medium. CaCl_2 additions (arrows) were 5 μmol each (final concn.). A typical experiment out of three is reported.

Figure 3 Relationship between P_i concentration and MgATP-dependent Ca^{2+} -loading capacity in brain (B), hepatoma (H) and platelet (P) microsomes

Microsomes (0.02–0.025 mg of protein/ml) were incubated in the medium for MgATP-dependent $^{45}\text{Ca}^{2+}$ uptake (see legend to Figure 1), including P_i at the indicated concentrations, for 1 h. The amounts of Ca^{2+} accumulated by microsomes are expressed on the basis of the protein (a) and water (b) contents of the microsomal fractions (see the Experimental section). Data are means $\pm$ S.E.M. of 4–6 experiments.

Table 1 Co-accumulation of $^{45}\text{Ca}^{2+}$ and $[\text{P}_i]$ in microsomal fractions from brain, hepatoma and platelets

Microsomes (0.02–0.025 mg of protein/ml) were incubated in the medium for MgATP-dependent $^{45}\text{Ca}^{2+}$ uptake (see the Experimental section) including $^{45}\text{CaCl}_2$ as a tracer and 3 mM P_i , or non-labelled Ca^{2+} and 3 mM P_i plus $[\text{P}_i]$ as a tracer, for 1 h. When included in the incubation medium, A23187 was 2 μM . Net values for $[\text{P}_i]$ accumulation are the difference between samples with and without A23187. Microsomal accumulation of $^{45}\text{Ca}^{2+}$ and $[\text{P}_i]$ associated with microsomes were measured as reported in the Experimental section. Data are means $\pm$ S.E.M. of 4–6 experiments.

Tissue	$^{45}\text{Ca}^{2+}$ accumulation (nmol/mg of protein)		$[\text{P}_i]$ associated with microsomes (nmol/mg of protein)		Net $[\text{P}_i]$ accumulation (nmol/mg of protein)
	– P_i	+ P_i	– A23187	+ A23187	
Brain	23.9 $\pm$ 1.3	104.4 $\pm$ 7.2	83.4 $\pm$ 9.8	30.3 $\pm$ 1.8	53.1
Hepatoma	10.2 $\pm$ 0.5	64.8 $\pm$ 4.6	67.3 $\pm$ 6.4	31.1 $\pm$ 5.9	36.2
Platelets	16.3 $\pm$ 1.1	155.1 $\pm$ 6.3	119.0 $\pm$ 3.3	33.6 $\pm$ 5.6	85.4

microsomes were also measured under other experimental conditions suitable to suppress Ca^{2+} pumping (results not shown), namely in the presence of excess EGTA (2 mM) or vanadate (1 mM).

The time course for $^{45}\text{Ca}^{2+}$ and $[\text{P}_i]$ uptake by brain microsomes is reported in Figure 4. During the second P_i -dependent phase, a net $[\text{P}_i]$ uptake paralleled $^{45}\text{Ca}^{2+}$ uptake,

Figure 4 Time course of MgATP-dependent microsomal $^{45}Ca^{2+}$ and $[^{32}P]P_i$ uptake

Brain microsomes (0.02–0.025 mg of protein/ml) were incubated in the medium for MgATP-dependent $^{45}Ca^{2+}$ uptake (see legend to Figure 1), including $^{45}CaCl_2$ and 3 mM P_i (○), or including non-labelled Ca^{2+} and 3 mM P_i plus $[^{32}P]P_i$ (●). Microsomal accumulations of $^{45}Ca^{2+}$ and $[^{32}P]P_i$ were measured as reported in the Experimental section. Net values for microsomal $[^{32}P]P_i$ accumulation are shown. They were obtained by subtracting $[^{32}P]P_i$ non-specifically associated with microsomes as measured in parallel samples including 2 μM A23187 to suppress Ca^{2+} uptake. Data are means $\pm$ S.E.M. of four experiments.

Figure 5 EGTA-induced effluxes of (a) $^{45}Ca^{2+}$ and $[^{32}P]P_i$ from $^{45}Ca^{2+}$ - or $[^{32}P]P_i$ -pre-loaded microsomes and of (b) $^{45}Ca^{2+}$ from microsomes pre-loaded with different amounts of $^{45}Ca^{2+}$

(a) Brain microsomes (0.02–0.025 mg of protein/ml) were pre-loaded with $^{45}Ca^{2+}$ or $[^{32}P]P_i$ in the presence of MgATP and 3 mM P_i for 1 h. After loading, EGTA (2 mM) was added, and microsomal $^{45}Ca^{2+}$ and $[^{32}P]P_i$ were measured by rapid filtration at the indicated time points. Net values for microsomal $[^{32}P]P_i$ content were calculated as reported in the legend to Figure 4. Data are percentages of $^{45}Ca^{2+}$ or $[^{32}P]P_i$ contents in microsomes at the time of EGTA addition. Means $\pm$ S.E.M. of three experiments are shown. (b) Microsomal $^{45}Ca^{2+}$ pre-loading and EGTA-induced efflux were performed as described above, except that different $^{45}Ca^{2+}$ loading values were obtained by including no P_i (○) or 1 (●), 2 (■) or 3 (▲) mM P_i in the incubation system. Values shown are nmol of Ca^{2+} /mg of protein. A typical experiment out of three is reported.

whereas no intravesicular $[^{32}P]P_i$ accumulation took place during the first rapid phase.

Passive fluxes of Ca^{2+} and P_i in microsomal vesicles

Two main components affect Ca^{2+} accumulation by microsomal vesicles: the rate of MgATP-dependent Ca^{2+} inward transport and the rate of passive Ca^{2+} back-flux. Therefore, the influence of

Figure 6 A23187-induced effluxes of $^{45}Ca^{2+}$ and $[^{32}P]P_i$ from $^{45}Ca^{2+}$ - or $[^{32}P]P_i$ -pre-loaded microsomes (a) and of Ca^{2+} from microsomes pre-loaded with different amounts of Ca^{2+} (b)

(a) Brain microsomes (0.02–0.025 mg of protein/ml) were pre-loaded with $^{45}Ca^{2+}$ or $[^{32}P]P_i$ in the presence of MgATP and 3 mM P_i for 1 h. After loading, A23187 (2 μM) was added, and microsomal $^{45}Ca^{2+}$ and $[^{32}P]P_i$ were measured by rapid filtration at the indicated time points. Net values for microsomal $[^{32}P]P_i$ content were calculated as reported in the legend to Figure 4. Data are percentages of $^{45}Ca^{2+}$ or $[^{32}P]P_i$ contents in microsomes at the time of A23187 addition. Means $\pm$ S.E.M. of four experiments are shown. (b) The release of Ca^{2+} by A23187 (4 μM) was measured by monitoring the free Ca^{2+} concentration in the incubation mixture by Ca^{2+} -sensitive electrodes. Brain microsomes (0.5–1.0 mg of protein/ml) were incubated in the presence of MgATP and 3 mM P_i . Different amounts of $CaCl_2$ (10–50 μM /ml) were included in the incubation system in order to load microsomal vesicles with different amounts of Ca^{2+} . At any 1–2 s time interval after A23187 addition, the amount of Ca^{2+} still present in microsomes was calculated as reported in the Experimental section. Values (nmol of Ca^{2+} /mg of protein) were used to draw the curves shown. A typical set of experiments out of two or three is reported.

co-accumulation of Ca^{2+} and P_i on the passive efflux of Ca^{2+} (and P_i) from microsomal vesicles was investigated.

Figure 5(a) shows the time course of $^{45}Ca^{2+}$ and $[^{32}P]P_i$ efflux from brain microsomal vesicles pre-loaded with $^{45}Ca^{2+}$ and $[^{32}P]P_i$ after inhibition of active Ca^{2+} inward transport by excess (2 mM) EGTA. Both ions are simultaneously and completely released within 1 h. Qualitatively similar results were obtained with the Ca^{2+} -pump inhibitor vanadate (1 mM; results not shown). In further experiments, the time course of $^{45}Ca^{2+}$ efflux by EGTA was investigated in (brain) microsomes which had been pre-loaded with different amounts of $^{45}Ca^{2+}$ in the presence of different concentrations of P_i (0, 1, 2 and 3 mM). By increasing the intravesicular Ca^{2+} (and P_i) loading, the inhibition of active Ca^{2+} transport by EGTA resulted in a proportionally slower release of Ca^{2+} (Figure 5b).

The P_i -dependent enlargement of microsomal Ca^{2+} -loading capacity therefore appears to be related to a relative decrease in the passive Ca^{2+} back-flux. This decrease, however, was very unlikely to be due to the formation of intravesicular insoluble Ca^{2+} - P_i complexes. In fact, the permeabilization of microsomal membrane to Ca^{2+} with the Ca^{2+} ionophore A23187 induced a rapid release of $^{45}Ca^{2+}$ and $[^{32}P]P_i$ from pre-loaded brain microsomes; at the shortest time interval of the ultrafiltration technique (20 s), most of the $^{45}Ca^{2+}$ and $[^{32}P]P_i$ was released (Figure 6a). Moreover, A23187-induced Ca^{2+} release was independent of the extent of Ca^{2+} pre-loading of microsomes, as observed by monitoring the time course of A23187-induced Ca^{2+} efflux by Ca^{2+} electrodes (Figure 6b). A similar and rapid ($t_{1/2}$ approx. 2 s; data from

Figure 7 Efflux of $^{45}\text{Ca}^{2+}$ and $[^{32}\text{P}]\text{P}_i$ from $^{45}\text{Ca}^{2+}$ - or $[^{32}\text{P}]\text{P}_i$ -pre-loaded microsomal vesicles induced by dilution of extravesicular P_i

Brain microsomes (0.5 mg of protein/ml) were loaded with $^{45}\text{Ca}^{2+}$ or $[^{32}\text{P}]\text{P}_i$ in the presence of MgATP and 3 mM P_i for 1 h. After loading, incubation were diluted 25-fold with the complete medium lacking P_i and the amount of $^{45}\text{Ca}^{2+}$ or $[^{32}\text{P}]\text{P}_i$ associated with microsomes was measured thereafter by rapid filtration. P_i concentration in diluted samples was 0.2–0.3 mM. Data are percentages of $^{45}\text{Ca}^{2+}$ or $[^{32}\text{P}]\text{P}_i$ associated with microsomes before dilution. Means $\pm$ S.E.M. of three experiments are shown.

Figure 8 InsP_3 -induced Ca^{2+} release in brain microsomes loaded with different amounts of Ca^{2+}

Ca^{2+} uptake and the subsequent InsP_3 -induced release of Ca^{2+} were monitored by measuring the free Ca^{2+} concentration in the incubation mixture with Ca^{2+} electrodes. Microsomes (MS; 1 mg of protein/ml) were incubated in the presence of MgATP and in the absence (a) or in the presence of 3 mM added P_i (b) at 37 °C under continuous stirring. CaCl_2 (three pulses, 10 nmol each; arrows) was added to the sample including P_i . InsP_3 and A23187 were 2 μM each (final concns.). A typical set of experiments out of 4–5 is reported.

Figure 6b) efflux time was found for brain microsomes preloaded with Ca^{2+} amounts ranging from 19 to 106 nmol/mg of protein. Analogous results (not shown) were also obtained with platelet and hepatoma microsomes.

Microsomes also released $^{45}\text{Ca}^{2+}$ and $[^{32}\text{P}]\text{P}_i$ when the extravesicular P_i concentration was lowered to values (< 0.2 mM) not able to stimulate Ca^{2+} accumulation appreciably. In fact, the dilution of preloaded brain microsomes in a P_i -free medium (but including MgATP) caused a concomitant efflux of $[^{32}\text{P}]\text{P}_i$ and $^{45}\text{Ca}^{2+}$ (Figure 7). Although the loss of $[^{32}\text{P}]\text{P}_i$ was almost complete, the presence of MgATP in the dilution medium

Figure 9 Correlation between P_i -stimulated MgATP-dependent Ca^{2+} accumulation and InsP_3 -induced Ca^{2+} release in brain, hepatoma and platelet microsomes and permeabilized platelets

Ca^{2+} uptake and the subsequent InsP_3 -induced release of Ca^{2+} were measured by monitoring the free Ca^{2+} concentration in the incubation mixture with Ca^{2+} electrodes. Microsomes (1–2 mg of protein/ml) or permeabilized cells (3–4 mg of protein/ml) were incubated in the presence of MgATP and 3 mM P_i (as potassium phosphate buffer, pH 7.2), at 37 °C under continuous stirring. Different amounts of CaCl_2 (10–50 $\mu\text{mol}/\text{ml}$) were included in the incubation system in order to load microsomal vesicles with different amounts of Ca^{2+} . Microsomes or permeabilized platelets were incubated until the free Ca^{2+} concentration of the medium was lowered to approx. 0.2 μM ; then InsP_3 (2 μM final concn.) was added. The amounts of Ca^{2+} accumulated and of Ca^{2+} released by InsP_3 were quantified as reported in the Experimental section. Data are means $\pm$ S.E.M. of 4–6, 3–5 and 3 experiments for brain, hepatoma and platelets respectively. Data for permeabilized platelets are means of two experiments.

allowed microsomes to retain a portion of the accumulated $^{45}\text{Ca}^{2+}$ which corresponded to the amount of Ca^{2+} accumulated by microsomes in the absence of P_i stimulation (see Figure 1).

Phosphate stimulation of active Ca^{2+} accumulation and InsP_3 -induced Ca^{2+} release

The InsP_3 -sensitive Ca^{2+} pool has been found to be associated with the microsomal fractions prepared from several tissues (e.g. [1–6]). The possibility that the P_i -dependent microsomal Ca^{2+} accumulation may result in an enlargement of the Ca^{2+} pool responsive to InsP_3 was therefore investigated. To this end, microsomes were pre-loaded with different amounts of Ca^{2+} (10–60 nmol/mg of protein) in the presence of MgATP and P_i (3 mM) by varying the concentration of total external Ca^{2+} and InsP_3 -induced Ca^{2+} release was measured by Ca^{2+} electrodes.

Figure 10 $InsP_3$ -induced releases of $[^{32}P]P_i$ (a) and Ca^{2+} (b) from brain microsomes pre-loaded with Ca^{2+} and $[^{32}P]P_i$

Microsomes (1 mg of protein/ml) were pre-loaded with $[^{32}P]P_i$ and Ca^{2+} in the presence of 3 mM MgATP (plus an ATP-regenerating system), 3 mM P_i (plus $[^{32}P]P_i$, 3 μ Ci/ml) and 64 μ M total Ca^{2+} (50 μ M added, plus 14 μ M already present as contaminant). In control experiments (lower traces, $\square$) a lower P_i -independent microsomal Ca^{2+} loading was accomplished under the same experimental conditions, except that no exogenous Ca^{2+} was added. After loading, heparin (H; 25 μ M final concn.), $InsP_3$ (3 μ M final concn.) and A23187 (ion.; 4 μ M final concn.) were added at the indicated time points (arrows). Microsomal Ca^{2+} content was measured with Ca^{2+} electrodes (see legend to Figure 9). $[^{32}P]P_i$ associated with microsomes was measured in 50 μ l samples of incubates by rapid filtration. A typical experiment out of 3 is reported.

Because of enlargement by P_i of Ca^{2+} -loading capacity, the microsomal fractions lowered the external free Ca^{2+} concentration to approx. 0.2 μ M, irrespective of the concentration of total external Ca^{2+} . Therefore the $InsP_3$ -induced Ca^{2+} release was evaluated at different steady-state levels of intravesicular total Ca^{2+} but at a fixed low concentration of the ambient free Ca^{2+} . Actually, low concentrations of extravesicular free Ca^{2+} appear to be required for the maximal Ca^{2+} -releasing effect of $InsP_3$ [1,2,6]. A typical experiment with brain microsomes is shown in Figure 8. It is evident that the increase in Ca^{2+} accumulation, depending on P_i stimulation, resulted in an increase in the amount of Ca^{2+} released by $InsP_3$.

As shown in Figure 9, the amounts of Ca^{2+} mobilized by $InsP_3$ linearly correlated with the amounts of Ca^{2+} accumulated by the microsomal fractions in the three non-hepatic tissues. A qualitatively similar correlation was also present in digitonin-permeabilized platelets (Figure 9c), although the percentage of the $InsP_3$ -releasable Ca^{2+} (31 %) was higher than that observed in platelet microsomes (17 %). In some experiments, it was verified that the kinetics of $InsP_3$ -induced Ca^{2+} release was similar ($t_{1/2} \leq 2$ s) in any fraction, independently of the amount of released Ca^{2+} .

The possibility that P_i anions are released by $InsP_3$ in microsomes preloaded with Ca^{2+} and P_i was also investigated. To this end, in some experiments where $InsP_3$ -induced Ca^{2+} release was measured with a Ca^{2+} electrode, $[^{32}P]P_i$ was included in the incubation mixture and microsome-associated radioactivity was also measured. In (brain) microsomes pre-loaded with Ca^{2+} under P_i stimulation, $InsP_3$ -induced Ca^{2+} release (Figure 10a, $\bullet$) was paralleled by a loss of microsome-associated $[^{32}P]P_i$. Subsequent addition of A23187 released vesicular Ca^{2+} almost completely and caused a marked loss of microsome-associated $[^{32}P]P_i$. Heparin addition suppressed both the release of Ca^{2+} and the loss of microsome-associated $[^{32}P]P_i$ induced by $InsP_3$, but it did not affect the A23187-induced release of Ca^{2+} and $[^{32}P]P_i$ (Figures 10a and 10b, $\circ$). Under experimental conditions suitable to load vesicles with low Ca^{2+} amounts independently of P_i stimulation (i.e. in the presence of low amounts of external Ca^{2+}), $InsP_3$, and subsequently A23187, mobilized vesicular Ca^{2+} while the levels of microsome-associated $[^{32}P]P_i$ were unmodified (Figures 10a and 10b,

lower curves). These levels likely reflected the non-specific binding of P_i to microsomes, as they were very similar to those observed after A23187-induced loss of $[^{32}P]P_i$ from microsomes efficiently pre-loaded with $[^{32}P]P_i$ (compare upper and lower curves of Figure 10a). In these experiments, dilution of the radioisotope with the unlabelled P_i continuously generated from ATP (high protein concentrations were employed) did not allow quantification of intravesicular P_i . Nevertheless, the observed variations in microsome-associated $[^{32}P]P_i$ indicated that P_i is indeed released by $InsP_3$. Qualitatively similar results (not shown) were also obtained in hepatoma microsomes.

DISCUSSION

In the present study we show that low concentrations of P_i , in the range of those reported to occur in the cytosol [25–30], favour Ca^{2+} uptake and storage, via Ca^{2+} and P_i co-accumulation, in ER vesicles derived from various non-hepatic tissues. These findings extend and confirm previous similar observation in liver [17] and skeletal-muscle reticular preparations [21].

We also show here that P_i stimulation of microsomal Ca^{2+} uptake is operative in the presence of low cytosol-like ambient free Ca^{2+} concentrations, and this further strengthens the possibility for a physiological role of cytosolic P_i in the homeostatic control of cytosolic Ca^{2+} by the ER.

It is worth considering that the amounts of Ca^{2+} accumulated by the microsomal fractions under P_i stimulation (see Figure 3b) are similar to those envisaged to be present *in vivo*. The concentration of 'total' Ca^{2+} in ER compartment(s), in fact, appears to be 5–50 mM when calculated on the basis of the total cellular Ca^{2+} [2,3], Ca^{2+} distribution among [2,3] and relative water space of [2] the different organelles. In addition, electron-probe microanalysis measurements of Ca^{2+} in the ER *in situ* of various tissues [8–10] are essentially in agreement with the microsomal loading values observed in the present study.

With respect to the mechanisms(s) involved in P_i stimulation of Ca^{2+} uptake, data obtained with $[^{32}P]P_i$ indicate that a co-uptake of Ca^{2+} and P_i occurs. In the presence of P_i , the microsomal Ca^{2+} uptake is biphasic, and co-uptake of Ca^{2+} and P_i ensues after an early P_i -independent active Ca^{2+} -uptake phase

has been accomplished. The ER Ca^{2+} pump is electrogenic and requires a compensating flow of counter-ions (such as K^+) to maintain electroneutrality [40]. This mechanism probably operates until the intravesicular steady-state concentration of free Ca^{2+} attained inhibits further Ca^{2+} pumping [41]. In the presence of ambient P_i , a subsequent co-uptake of Ca^{2+} and P_i may allow electroneutrality and 'buffering' of intravesicular free Ca^{2+} , which prevents Ca^{2+} inhibition of the pump. The lack of P_i accumulation during the early phase of Ca^{2+} uptake could be due to a poor permeability of the microsomal membrane for P_i anions (at least compared with that for K^+). Preliminary experiments (R. Fulceri, G. Bellomo and A. Benedetti, unpublished work) indicate that external P_i do not passively equilibrate with the intravesicular water space of microsomes from brain, hepatoma and platelets.

Data concerning Ca^{2+} and P_i efflux from pre-loaded microsomes indicate that membrane permeabilization to Ca^{2+} (by A23187 and InsP_3), inhibition of Ca^{2+} inward transport (by excess EGTA or vanadate), or lowering of external P_i result in the concomitant mobilization of stored Ca^{2+} and P_i ions. Increases in membrane permeability to Ca^{2+} cause a rapid (a few seconds) release of Ca^{2+} and P_i independently of the microsomal Ca^{2+} (and P_i) content. Therefore, intravesicular Ca^{2+} - P_i insoluble complexes are not formed. Instead, intravesicular Ca^{2+} complexation or buffering by P_i allows a prompt mobilization of the stored ions. On the other hand, the passive back-flux of stored Ca^{2+} which follows inhibition of Ca^{2+} inward transport lasts much longer (several minutes) and does not proportionally increase as the intravesicular Ca^{2+} (and P_i) content becomes higher. Both intravesicular buffering of P_i and/or saturation of microsomal Ca^{2+} -efflux pathway(s) may contribute to the relatively low Ca^{2+} leakiness in Ca^{2+} - P_i -loaded microsomes. Direct measurements of intravesicular free Ca^{2+} are needed to clarify this point.

In terms of cell physiology, Ca^{2+} stored in the ER compartment(s) should be somehow 'buffered' but rapidly exchanged with the cytosolic compartment. Intracellular storage of Ca^{2+} and P_i may well satisfy such conditions.

A relevant finding of the present study is that the InsP_3 -mobilizable Ca^{2+} pool is responsive to P_i stimulation. Actually, the P_i -dependent enlargement of microsomal Ca^{2+} content results in a proportional enlargement of the InsP_3 -sensitive Ca^{2+} pool in the three non-hepatic microsomal fractions and in digitonin-permeabilized platelets. Noticeably, stored P_i ions are also mobilized by InsP_3 concomitantly with Ca^{2+} .

Differently from the three non-hepatic cells investigated here, we previously observed [17] that in (permeabilized) hepatocytes physiological P_i concentrations are unable to affect the InsP_3 -sensitive Ca^{2+} pool(s). However, an enhancement of hepatic InsP_3 -sensitive Ca^{2+} pool has been observed when microsomal subfractions or permeabilized hepatocytes were stimulated by glucose 6-phosphate [5]. This could be ascribed to the existence of different sensitivities to InsP_3 , P_i and glucose 6-phosphate in the various sub-compartments of liver ER.

Collectively, the data reported here indicate that parallel and inter-related fluxes of Ca^{2+} and P_i ions between ER and cytosolic compartments may occur. In this respect, the possibility that fluctuations in cytosolic P_i concentrations may affect Ca^{2+} translocations across the membrane of reticular store(s) exists and requires further investigations.

This work was supported by grants from the Italian Ministry for University & Research and from the Italian Research Council (CNR), Strategy Project "Oncologia".

We thank Dr. Toni Scarpa (Case Western Reserve University, Cleveland, OH, U.S.A.) for helpful advice. We also thank Mrs. Cristina Pallini for technical assistance.

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